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A SIMPLE METHOD FOR DETECTING MASTITIS STREPTOCOCCI IN MILK

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INTRODUCTION

During recent years increasing care has been exercised, in procuring market-milk supplies, to obtain milk that meets certain sanitary requirements. Rules can be formulated for care and handling of utensils and care of milk after its production, but these do not prevent the production of abnormal milk by the cow.

The occurrence of abnormal milk is generally due to bacterial action. Changes in the milk so caused may originate either outside or inside the udder. A species of bacteria found in the udder, which is the cause of most cases of mastitis and the resultant abnormality in the milk, is *Streptococcus agalactiae* (*mastitidis*). This organism is said to be present in from 90 to 95 percent of streptococcic mastitis. Detection of the presence of this organism and of the stage of abnormality of the milk is sometimes a problem. In severe cases of infection the milk is so changed that flakes or clots, which can be readily detected, appear in the milk; but in chronic cases the product may be changed in composition without its appearance being altered.

Nearly all the tests usually used yield certain information about changes which have taken place in the udder and milk. However, none of them gives more than one phase and several tests must be used in conjunction with each other in order that an opinion may be formed as to the abnormality of the milk. Again, most of the tests are delicate, require a laboratory with considerable equipment, and must be made by a trained technician.

Any satisfactory method for detecting mastitis streptococci in milk must not only be accurate, but, to be readily used in determining the

[1] Mr. Hotis died July 19, 1935. From the records he left, the junior author prepared this Circular.

sources of infection among large numbers of animals, it must require a minimum of equipment, materials, time, and labor, in testing individual samples of milk directly from one or more quarters of the udder.

REVIEW OF THE LITERATURE

Various methods have been devised for the detection of *S. agalactiae* and changes in the composition of milk. Some of these are direct microscopic examination, including the determination of the presence of streptococci and the number of leucocytes; bacteriological culture methods for determining the number of causative organisms; strip-cup test; chloride test; brom-thymol-blue test; catalase test; and physical examination of the udder.

The direct microscopic method, brought out by Breed (*2*),[2] is a rapid means of examining the milk and will detect sources of infection carrying large numbers of streptococci but does not necessarily indicate the stage of the abnormality of the milk.

The leucocyte-count method reported by Prescott and Breed (*7*) is a direct microscopic examination of milk for the purpose of determining the number of white blood corpuscles in the milk. The number gives indication of the severity of the infection in the udder.

Under bacteriological culture methods there are the Petri-plate method, microscopic examination of the incubated milk sample, and the Burri (*3*) slant method. With the Petri-plate method it is usually the practice to prepare special media to which defibrinated horse blood is added. The Burri slant method (named after its originator) requires the use of a standardized loop with which the milk is smeared over the slant. The incubated-sample method is used for determining carriers of small numbers of streptococci and consists in incubating fresh, aseptically drawn, samples of milk for 12 to 24 hours at 37° C. All the bacteriological methods are used merely for determining the presence or absence of *S. agalactiae*.

The strip-cup test was first used by Moak (*6*) and is one of the most practical means available for use on the farm to detect such abnormal conditions in the milk as clots, etc.

The chloride test, developed by Hammer and Bailey (*4*), is used to detect an increase of salts which filter through diseased udder tissues into the milk. The test is very useful for indicating infected udders, but because of its delicacy its use is confined largely to research laboratories.

The brom-thymol-blue test was developed at the New York State Agricultural Experiment Station. It indicates the alkalinity of milk. Milk when freshly drawn from udders in which there is an infection is more alkaline than milk from normal udders, because of the infusion of blood plasma into it.

The ability of the enzyme catalase to break down hydrogen peroxide into water and hydrogen led to the development of the catalase test by Trommsdorff (*8*).

Physical examination of the udder as recently developed (*9*) has provided a practical and fairly accurate means of detecting mastitis without the aid of laboratory measures. By palpating the udder after milking, in order to determine the presence or absence of

[2] Italic numbers in parentheses refer to Literature Cited, p. 6.

fibrosis or indurations in the glandular tissue, an experienced operator can determine whether the animal is affected with mastitis.

THE HOTIS TEST AND RESULTS OF ITS USE

This circular describes a method developed by the senior author in the laboratories of the Bureau of Dairy Industry during 1933–35, which appears to approach fulfillment of the requirements mentioned above. This method will be called the Hotis test in recognition of the senior author who devised and developed it.

The organism *S. agalactiae* has a very definite growth characteristic when grown as pure culture in infusion broth; i. e., heavy growth in the bottom and up the side of the test tube. In working with milk samples the senior author noted that when some samples were incubated in small-bore glass tubes for a period of 24 hours there would appear on the side small round flakes which were seemingly denser than the surrounding medium. Having noted the characteristic growth in infusion broth and knowing that milk is a natural habitat of the organism, he examined this colonylike growth in milk and found it to be *S. agalactiae.* However, such growth was somewhat hard to see, so means were sought by which the growth could be recognized more easily.

Klimmer (*5*), in his serum alkali albuminate saccharose agar, added a saturated aqueous solution of brom-cresol-purple. He stated that on this agar *S. agalactiae* colonies in 24 hours are a dark yellow surrounded by a small cloudy border and a larger bright-yellow halo. Baker and Van Slyke (*1*) had shown that brom-cresol-purple when added to milk possesses properties which make it an indicator for the colorimetric determination of hydrogen-ion concentration in milk. From these observations, the senior author concluded that a change in color after the addition of this dye to the milk would indicate acid production and possibly permit easier recognition of the flakes, thus facilitating the identification of *S. agalactiae.*

The Hotis test, therefore, consists in adding 0.5 cc of a sterile 0.5-percent aqueous solution of bromo-cresol-purple (dibromorthocresolsulphonphthalein) to 9.5 cc of milk previously measured into a sterile test tube. After the tube is inverted several times to mix the contents, the sample is incubated at 37.5° C. for 24 hours and the results are then observed. A mixed sample of milk, consisting of an equal quantity from each quarter of the udder, or samples from individual quarters may be used for the test. However, the individual quarter sample is probably more accurate, especially when streptococci are present in small numbers in only one quarter.

Immediately after the milk and indicator have been mixed, a deep-purple color results. The color is due to the fact that the acidity of milk (pH 6.3 or over) is low and therefore in the upper range of the indicator (pH 5.2, yellow to pH 6.8, purple). If streptococci are present, the color changes from purple to a yellow shade during incubation as a result of the production of acid from lactose by these organisms. In addition to this change, if *S. agalactiae* is present, small flakes or balls of growth, from 0.5 to 4 mm in diameter, usually form on the side of the tube. These growths are apparently composed of milk curd and entwined chains of *S. agalactiae*, which is the predominant species found in streptococcic mastitis. Their occurrence

has been observed frequently in incubated milk to which no indicator has been added, but in this test they are seen more readily because, as a rule, they appear as canary-yellow spots attached to the side of the tube and immediately surrounded by a halo of the same color, which in turn gradually merges with the darker background of the column of milk. The number of flakes varies from several to countless and the size from pin point, when there are many flakes, to about 4 mm in diameter when only a few are present. When *S. agalactiae* is present in the sample, it is rare that no flakes or clumps are noted.

Plate 1 shows typical color reactions and the characteristic flakes.

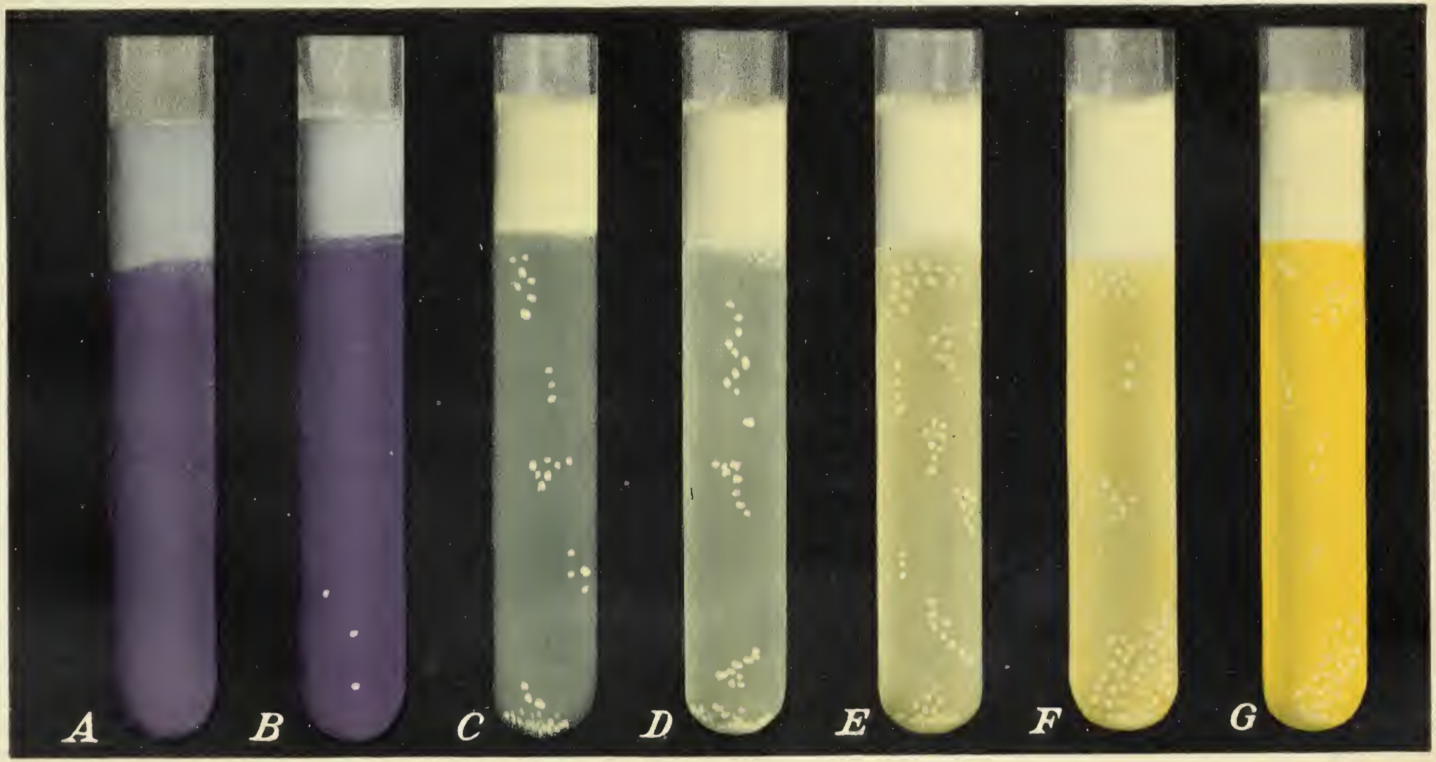
Various shades of color are encountered in tests which may be considered suspicious or positive. These range from a blue gray or a distinct olive color through yellowish green and greenish yellow to a pronounced yellow or golden hue. When the first-mentioned colors occur, an additional 24 hours' incubation will usually bring out the yellow color. However, if the indistinct shades are associated with the occurrence of flakes on the side of the tubes, which is very often the case, no further incubation is necessary to indicate a positive result. A little experience with the test will enable the operator to recognize these points without difficulty.

During the course of this work 753 samples of milk were subjected to the Hotis test for the detection of mastitis streptococci and also to the blood-agar plate method of determining the presence of these organisms. The majority of these samples were from individual quarters and the remainder were composite samples taken directly from the four quarters of the udder. Table 1 shows the samples classified as positive or negative as indicated by the Hotis test and also the number and percentage of the samples shown to contain mastitis streptococci by the blood-agar method.

TABLE 1.—*Results of examining 753 samples of milk by the Hotis test and by the blood-agar method to determine the presence or absence of mastitis streptococci*

Hotis test				Presence (+) or absence (−) of mastitis streptococci on blood-agar plates
Appearance of samples after incubation	Reaction indicated by appearance	Samples classified according to appearance and reaction		
		Number	*Percent*	
Flakes, and typical color change	+	560	74.4	+
No flakes, but typical color change	+	108	14.4	+
		25	3.3	−
No flakes, and no color change	−	13	1.7	+
		47	6.2	−

Positive reactions to the Hotis test, showing both the typical color change and the flakes on the side of the tube, occurred in 560 samples in which the blood-agar plates showed mastitis streptococci. Positive color reactions without the characteristic flakes occurred in 108 additional samples in which the blood-agar plates showed streptococci, and in 25 in which no streptococci were found. Negative reactions to the Hotis test occurred in 60 samples, only 13 of which showed streptococci on blood-agar plates.



Appearance of samples after 24 hours' incubation: *A*, negative test. The color remains unchanged, no flake formation; *B*, positive test. The small yellow flakes are masses of streptococci. The deeper purple color is due to a high pH (unusual); *C*, *D*, *E*, *F*, *G*, positive tests representative of the several characteristic reactions.

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As indicated in table 1 a total of 668 samples were classified positive both by the Hotis test and by the blood-agar method, and 47 were classified as negative. In other words the two methods were in perfect agreement for 715, or 95 percent, of the 753 samples tested. This figure may be considered an approximate measure of the accuracy of the Hotis test, if the results indicated by the blood-agar plates are assumed to be 100-percent accurate.

It is not possible at this time to offer a definite explanation for the variation in colors, or for the absence of flakes in some of the positive tests. While the Hotis method was being developed, blood-agar plates were made in order to determine the presence and the number of streptococci in the milk samples. There was some correlation between the number of organisms and the color of the sample as well as the number of flakes, but it was not sufficiently close to explain entirely the variation that occurred. There is some possibility that an investigation of the lactose content, pH value, and change in other constituents of the sample, together with the bacterial count, might yield the information. However, since the method was designed primarily to detect the presence of *S. agalactiae* in milk, very little work has been done to explain these variations.

Shortly after work on the test was started it appeared that determination of the quantity of sediment or growth produced in the sample during the incubation period might be informative. Accordingly, the tests were set up in sterile centrifuge tubes, graduated to 10 cc. After incubation the tubes were centrifuged for 5 minutes at about 2,000 revolutions per minute and the quantity of sediment was read in cubic centimeters from the graduations on the tube. The results were encouraging but not close enough to permit formulation of definite conclusions. One difficulty was the early coagulation of the milk in some samples, which tended to obstruct the descent of the sediment. This condition appeared to be due either to the presence of micrococci or to an overwhelming number of streptococci in the sample.

In order to obtain clear-cut results with the test it is highly desirable to collect the milk directly from the animal and as aseptically as possible. When the sample is taken from the pail or in a manner to permit gross contamination with rods, micrococci, and saprophytic streptococci, a positive reaction may be masked by proteolysis or alkali formation, or both, and occasionally a false positive result may ensue from contamination with lactic-acid bacteria. All these conditions have been encountered in the course of this work. The method as constituted at present, therefore, is not suitable for the examination of milk from cans or at receiving stations.

Advantages of this test are: (1) Only a small amount of equipment is required, such as a supply of sterile test tubes 12 mm in diameter, racks for tubes, sterile brom-cresol-purple indicator, a sterile pipette for adding the indicator, and an incubator set at 37.5° C.; (2) the milk sample having been collected in a sterile tube or bottle, it may be poured into the test tube graduated for 9.5 cc, thus obviating the use of a pipette for each sample and increasing the number of tests that can be set up in a given time; and (3) the milk serves as the culture medium and inasmuch as the brom-cresol-purple is apparently bacteriostatic for some of the udder micro-

cocci, at least for the first 24 hours, very little inaccuracy is experienced from this source when proper precautions have been observed in collecting the sample.

SUMMARY

A simple method for the detection of mastitis streptococci in milk that can be readily used on large numbers of samples and that requires but little equipment, is described. This test is designated as the Hotis test in recognition of the work of the senior author.

The method consists in adding 0.5 cc of a sterile 0.5-percent aqueous solution of brom-cresol-purple to 9.5 cc of milk carefully collected directly from the animal. After the sample is mixed it is incubated for 24 hours at 37.5° C. and the results are observed.

A characteristic change in the color of the sample after incubation together with the occurrence of flakes or balls of growth indicates the presence of *S. agalactiae.*

Examination of 753 samples of milk by the Hotis test and by the blood-agar method showed the two tests to be in perfect agreement for 715, or 95 percent, of the samples. This figure may be considered an approximate measure of the accuracy of the Hotis test, if the results indicated by the blood-agar plates are assumed to be 100-percent accurate.

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